The Heat-Shock Response: Its Variation, Regulation and Ecological Importance in Intertidal Gastropods (genus *Tegula*)[1]

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SYNOPSIS. **The enhanced synthesis of heat-shock proteins (hsps), called the heat-shock (or stress) response, is activated when environmental stress denatures proteins. Hsp synthesis is activated at the upper temperatures of an organism's thermal range and is therefore thought to be critical for enhancing thermal tolerance limits in ectothermic animals. Here I show that the two temperate sister species *T. brunnea* and *T. montereyi* that occupy the subtidal and low-intertidal zone differ from the low- to mid-intertidal *T. funebralis* (and the subtropical mid-intertidal *T. rugosa*) in (i) heat tolerance, (ii) the onset temperature of their main hsp, hsp70 (70 kDa), (iii) the temperature of maximal hsp70 synthesis, (iv) the upper temperature of hsp synthesis, and (v) the recovery from a thermal stress typical for the mid-intertidal zone. A regulatory model in which hsps themselves regulate their own transcription and synthesis through a negative autoregulatory feedback mechanism can explain acclimation-induced but not interspecific variation in the onset temperature of hsp70 synthesis. Transplanting species across their vertical distribution limits showed that interspecific differences in the stress response are likely to prevent species occurring lower from inhabiting sites higher in the rocky intertidal zone. Endogenous levels of a hsp of a molecular mass of 72 kDa, hsp72, changed little with heat stress in a species' native thermal environment. The results therefore confirm the importance of interspecific differences in the stress response for setting limits to an organism's thermal environment. However, the role of hsps as short-term indicators of sublethal heat stress within a species' native thermal environment may be limited without a better understanding of their functional and regulatory characteristics.**

INTRODUCTION

The rocky intertidal zone has long been used as an ideal "natural laboratory" to study a basic ecological question: how are physical and biological factors determining the distribution (*e.g.,* the vertical distribution) limits of organisms? After physiologists first emphasized the role of physical factors in setting at least the upper limits of vertical distribution patterns in marine invertebrates that inhabit the intertidal zone, ecologists showed that biological factors, *e.g.,* predation and competition, were important in setting the lower limits (for brief historical overview see Tomanek and Helmuth, 2002). Several studies showed that both factors are not acting independently of each other and that their role is not limited to the extremes of rocky shores (Wethey, 1983; 1984; Sanford, 1999). Recently, physiologists have taken advantage of advances in biochemistry and molecular biology that equip them with new tools to assess the ecological importance of physiological limits to sublethal physical stress, in particular heat stress. Here I present studies that have used heat-shock proteins (hsps) as indicators for sublethal thermal stress and that have elucidated the importance that the evolutionary (interspecific) variation in the heat-shock response may have on setting limits to vertical distribution patterns.

The heat-shock or stress response is activated in response to environmental and physiological stress that denatures proteins. It is characterized by the preferential synthesis of heat-shock (or stress) proteins that stabilize proteins, refold partially unfolded proteins and detect proteins that are irreversibly damaged under stressful conditions. Under non-stressful conditions hsps facilitate the correct folding of proteins during translation and, therefore, belong to a group of proteins called molecular chaperones (Lindquist, 1986; Lindquist and Craig, 1988; Parsell and Lindquist, 1994; Ellis, 1996; Feige *et al.*, 1996; Frydman, 2001; Hartl and Hayer-Hartl, 2002). Thus, in an ecological context, hsps may serve as potential indicators for protein synthesis as well as for reversible protein denaturation. The functions of hsps under stressful conditions are viewed as important in setting an organism's resistance to heat stress.

Species that occupy widely varying thermal niches have been shown to differ in their heat-shock responses (for review see Feder and Hofmann, 1999), but few of these studies established how this variation relates to the actual temperatures that these species experience under natural conditions. Furthermore, only few studies went beyond establishing species differences in the stress response and studied the variation in acclimatory plasticity that distinguishes species from diverse thermal environments (Dietz and Somero, 1992; Roberts *et al.*, 1997; Tomanek and Somero, 1999). The interspecific and acclimation-induced variation of hsp expression may be regulated mainly through the equilibrium between the heat-shock transcription factor-1 (HSF1) and several hsps which act as repressors of HSF1 activation, an interaction that has been called the "cellular thermometer" (DiDomenico *et al.*, 1982; Craig and Gross, 1991; Lindquist, 1993; Morimoto, 1998). Under non-stressful conditions several hsps

bind to the HSF1 and thereby repress its activation. Under stressful conditions hsps preferentially bind to denaturing proteins and this leads to the release of HSF1, which subsequently can activate hsp synthesis (Fig. 3; for further details see below and Tomanek and Somero, 2002). Several studies have shown that acclimation/acclimatization-induced variation in the induction (or onset) temperature of the heat-shock response is likely due to changes in endogenous levels of hsps that repress HSF1 activation, supporting the importance of the "cellular thermometer" model of transcriptional regulation of hsp expression for explaining such variation (Dietz and Somero, 1992; Roberts *et al.*, 1997; Tomanek and Somero, 1999, 2002; Buckley *et al.*, 2001).

Ultimately, I am interested in the ecological importance of interspecific and acclimation-induced variations in the stress response. Studies that elucidate the ecological importance of the stress response have to take into account the different roles of hsps as indicators for protein synthesis (or growth potential) and reversible protein denaturation. Furthermore, comparing changes in the level of hsps in individuals from different locations that vary either in terms of environmental stress or in nutrient levels can help to elucidate the ecological importance of the differing hsp functions (Dahlhoff and Menge, 1996; Dahlhoff *et al.*, 2001; L. Tomanek and E. Sanford, unpublished data). Field studies that relate environmental inputs and hsp levels should provide us with a better assessment of under what temporal and experimental conditions hsps can serve as reliable indicators of ecologically relevant stress.

The Intertidal Gastropod Genus *Tegula*

Here I want to address several of these questions by presenting an in-depth example of work that I conducted on a group of marine gastropod species that are closely related and that occupy widely varying thermal environments (Riedman *et al.*, 1981; Watanabe, 1984; Hellberg, 1998). The temperate mid- to low-intertidal *T. funebralis* experiences higher maximum temperatures (between 33°C and 35°C) in the field than the two congeners and sister species (*T. brunnea* and *T. montereyi*) that occupy the low-intertidal to subtidal zone (24°C). *T. funebralis* also experiences greater changes in body temperatures during low tide periods (19°C *versus* 7°C). Subtropical mid-intertidal *Tegula* congeners experience even greater temperatures than temperate mid-intertidal species, *e.g., T. rugosa* (sister species to *T. funebralis*) that is limited to the northern part of the Gulf of California (for temperatures of a species that inhabits the same thermal environment see Dietz and Somero, 1992). These observations in the field are paralleled by interspecific differences in thermotolerance (LT_{50}): the two sister species from the low-intertidal to subtidal zone (*T. brunnea* and *T. montereyi*) die at a lower temperature (36°C) than the mid- to low-intertidal *T. funebralis* (42.5°C; Tomanek and Somero, 1999). Thus, the genus provides an excellent system to study the following three questions: What are the interspecific and acclimation-induced variations in the heat-shock response? How is this variation in the stress response regulated? How relevant is this variation under natural conditions?

To answer these questions I first acclimated several *Tegula* species to 13°C, 18°C and 23°C in the laboratory and subsequently measured the *de novo* synthesis of hsps in gill tissue at a common temperature following exposure to a wide range of incubation temperatures (Fig. 1; Tomanek and Somero, 1999). These acclimation temperatures represent typical sea surface temperatures for the temperate *Tegula* congeners, with the exception of 23°C, which is close to the upper thermal limits of *T. brunnea* and *T. montereyi*. In addition to comparing the species over a wide range of incubation temperatures after a single time point of recovery, I quantified hsp synthesis over a 50 hr time period after an exposure to a temperature (30°C) that is frequently experienced by the mid-intertidal *T. funebralis* but is close to lethal for the subtidal to low-intertidal *T. brunnea* (Tomanek and Somero, 2000). I also quantified endogenous levels of hsp70 and hsp90, the two main repressors of the heat-shock response, as well as of HSF1 from these laboratory-acclimated snails to test several predictions of the transcriptional regulatory model of hsp expression for explaining the variation I detected (Tomanek and Somero, 2002). Finally, I tested predictions I made from my laboratory results, in regard to the importance of the variation that I detected under natural conditions, by transplanting the subtidal to low-intertidal *T. brunnea* into the mid-intertidal zone and quantifying endogenous levels of hsp70 isoforms (L. Tomanek and E. Sanford, unpublished data).

Interspecific and Acclimation-Induced Variation in the Heat-Shock Response

The autoradiographs in Figure 1 show patterns of newly synthesized proteins in gill tissues during 4 hr at a common temperature of 13°C following incubation to temperatures from 13°C to 36°C. With increasing incubation temperature the preferential synthesis of only a few proteins becomes apparent. Among the more dominant proteins that can be detected with one-dimensional polyacrylamide electrophoresis are hsps of a molecular mass of 38, 70, 77 and 90 kDa. The onset temperature (T_{on}) of the most prominent hsp, hsp70, differs between the subtidal and low-intertidal *T. brunnea* (and *T. montereyi*, data not shown) and the mid-intertidal *T. funebralis* by 3°C in 13°C-acclimated specimens (24°C *versus* 27°C, respectively; Figs. 1 and 2). 13°C-acclimated specimens of all three temperate species differed not only in (i) the onset temperature of hsp70 synthesis (T_{on}), but also in (ii) the temperature of maximal hsp70 synthesis (T_{peak}) as well as (iii) the upper thermal limits of hsp synthesis (T_{off}) according to the species' heat tolerance (Fig. 2; Tomanek and Somero, 1999). Furthermore, 23°C-acclimated specimens of the subtropical mid-intertidal *T. rugosa*

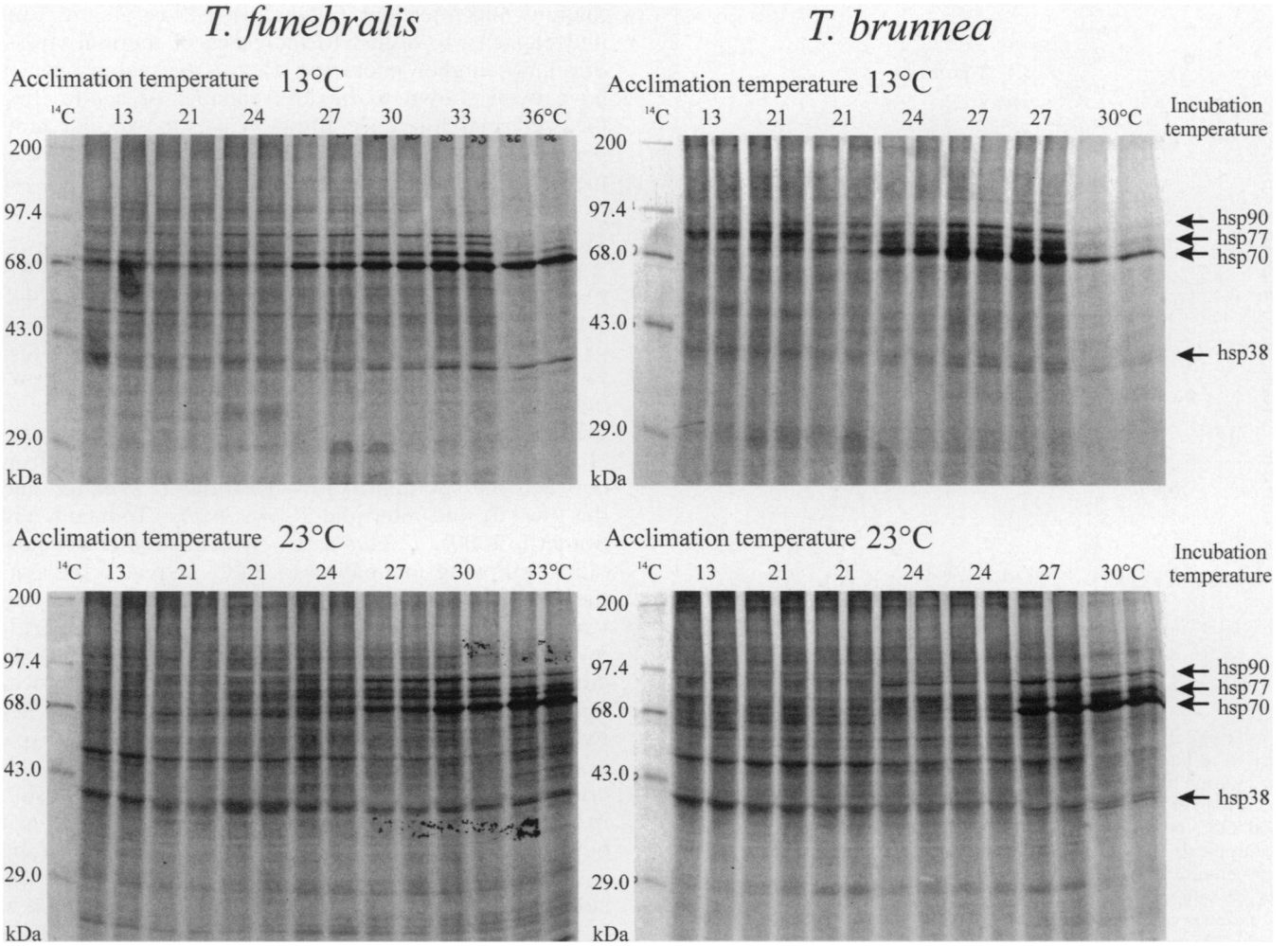

FIG. 1. Autoradiographs of newly synthesized proteins at different incubation temperatures (through incorporation of ^{35}S-labeled methionine/cysteine) in gill tissues of *Tegula funebralis* and *T. brunnea* acclimated to either 13°C or 23°C. For each incubation temperature, duplicates of a gill sample from a single snail was loaded on a 10% SDS-polyacrylamide gel. ^{14}C-markers are assigned a molecular mass in the left lane of each graph. Lanes were loaded with 500×10^3 counts min^{-1} and exposed for 8 hr to pre-flashed X-ray film (modified after Tomanek and Somero, 1999).

showed even higher temperatures for T_{on}, T_{peak} and T_{off} than its temperate mid-intertidal congener *T. funebralis*. Thus, interspecific differences in T_{on}, T_{peak} and T_{off} show a consistent rank order with the thermal environment (subtidal *versus* mid-intertidal) on a local scale among temperate species as well as between mid-intertidal *Tegula* congeners that differ in their latitudinal distribution range (temperate *versus* subtropical; Tomanek and Somero, 1999).

Interspecific differences in how frequently the heat-shock response is activated are strongly related to the vertical position in the rocky intertidal zone: first, *T. brunnea* and *T. montereyi* rarely experience temperatures as high as their T_{on} for hsp70 synthesis (24°C), even during extreme midday low tides. In contrast, *T. funebralis* and *T. rugosa* both frequently experience temperatures above their T_{on}'s, 27°C and 30°C, respectively, Thus, mid-intertidal congeners are likely to activate the heat-shock response more frequently than subtidal *Tegula* species. Other interspecific differences also relate to vertical position: T_{off}'s of the subtidal *T. brunnea* and *T. montereyi* (between 30°C to 33°C) are well within the temperature range that the mid-intertidal *T. funebralis* frequently experiences. This suggests that both subtidal species would be unable to synthesize hsps in the mid-intertidal zone, the thermal niche that is occupied by *T. funebralis*, at least during low tides on hot days. The upper temperature of protein synthesis (T_{off}) may therefore prevent *T. brunnea* and *T. montereyi* from inhabiting the mid-intertidal zone. Although I cannot exclude that T_{off} is simply a consequence of death (see below), the decline of hsp synthesis with increasing thermal stress (above T_{peak}) indicates that hsp synthesis itself is limited by thermal stress. Furthermore, T_{off}'s of both mid-intertidal *Tegula* species are only a few degrees (2°C to 3°C) above the highest temperatures that they experience in the field and are not subject to acclimatory adjustments. Thus, *T. funebralis* lives close to its thermal limit of protein synthesis. Small increases in extreme temperatures due

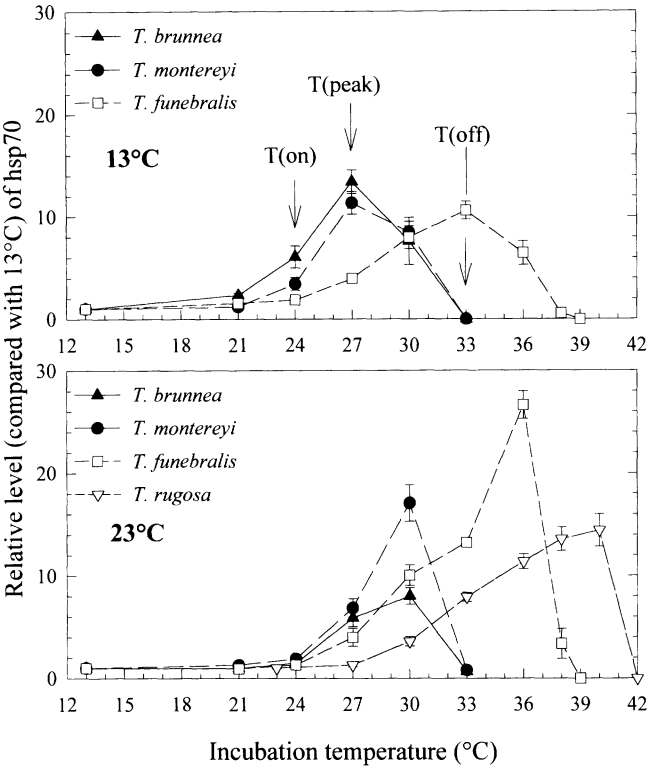

FIG. 2. Relative induction (compared to the 13°C control group) of hsp70 in the three temperate *Tegula* congeners *T. funebralis* (low- to mid-intertidal zone), *T. brunnea* and *T. montereyi* (both subtidal to low-intertidal zone) after laboratory acclimation at 13°C, 18°C and 23°C for 30–34 days, and relative induction for 23°C-acclimated subtropical *T. rugosa* (mid-intertidal zone). Indicated are the onset temperature (T_{on}), the temperature of maximal induction (T_{peak}) and the cessation temperature (T_{off}) of hsp synthesis for *T. brunnea* and *T. montereyi* only (for further details see text). Values are mean ± 1 S.E.M., $n = 5$ for all data points except 13°C-acclimated *T. funebralis* at 36° ($n = 4$) and 23°C-acclimated *T. funebralis* at 33°C and *T. brunnea* at 13°C ($n = 4$; modified after Tomanek and Somero, 1999).

to global warming may therefore be enough to stress these animals beyond their thermal limits (Sagarin *et al.*, 1999). The potential importance of T_{off} in setting thermal tolerance limits is suggested further by the observation that whole snails of these species do not survive exposure to temperatures at which we observed the cessation of hsp as well as general protein synthesis (Tomanek and Somero, 1999).

With acclimation to warmer temperatures (23°C) T_{on} shifted from 24°C to 27°C in *T. brunnea* and *T. montereyi* but stayed at 27°C in *T. funebralis*. Thus, the more heat-sensitive *T. brunnea* and *T. montereyi* showed a greater acclimatory plasticity in T_{on} of hsp70 synthesis than the more heat-tolerant *T. funebralis*. Other hsps that are synthesized at lower levels than hsp70 showed similar (*e.g.*, hsp38) and opposite (*e.g.*, hsp90) patterns of acclimatory plasticity. Acclimatory changes in T_{on} have been shown to occur naturally on a seasonal basis in intertidal fish and mussels (Dietz and Somero, 1992; Roberts, *et al.*, 1997). However, the lack of a shift in T_{on} of hsp70 synthesis in the heat-tolerant mid-intertidal *T. funebralis* illustrates its limited capacity to adjust to increases in thermal stress. Similarly, higher occurring *Petrolisthes* crab species have been shown to be less capable of acclimating their thermal tolerance limits (LT_{50}) to warmer temperatures than lower occurring ones (Stillman and Somero, 2000).

Time course of heat-shock protein synthesis

Due to the predictable temporal occurrence of thermal stress in the rocky intertidal zone during midday low tides it is necessary to consider the time course of the heat-shock response to elucidate further the ecologically important variation between species. Following the time course of hsp synthesis after exposure to thermal conditions that are typical for the mid-intertidal zone (30°C) I observed widely differing responses between the subtidal to low-intertidal *T. brunnea* and the low- to mid-intertidal *T. funebralis* (Tomanek and Somero, 2000). *T. funebralis*, which showed no mortality following incubation to 30°C, activated the synthesis of hsps immediately, reached maximal levels between 1–3 hr into recovery, and returned to prestress levels by 6 h (except for the synthesis of hsp90). In contrast, *T. brunnea*, for which 30°C represents a near lethal exposure, activated the synthesis of hsps between 2–14 hr into recovery (depending on the hsp), reached maximal levels between 15–30 hr and recovered to prestress levels 50 hr and 30 hr after exposure in case of hsp90 and hsp77, respectively. *T. brunnea* never recovered to prestress levels of synthesis in the cases of hsp70 and hsp38, even after 50 hr. These results illustrate that *T. funebralis*, but not *T. brunnea*, is able to recover from a thermal exposure typical for the mid-intertidal zone within the time between consecutive midday low tides.

In summary, the subtidal to low-intertidal *T. brunnea* and *T. montereyi* differ from the low- to mid-intertidal *T. funebralis* (and the subtropical *T. rugosa*) in (i) heat tolerance, (ii) the onset temperature of hsp70 (also hsp38 and hsp90) synthesis (T_{on}), (iii) the temperature of maximal hsp70 synthesis (T_{peak}), (iv) the upper temperature of hsp (and protein) synthesis (T_{off}), (v) rate of hsp synthesis following a 30°C exposure and (vi) the duration of hsp synthesis. Despite being different between species, some of these traits of the heat-shock response changed with acclimation to warm temperatures (from 13°C to 23°C), whereas others did not change at all (most notably T_{off}). Interspecific variation in the stress response has also been found in intertidal limpets of the genus *Collisella* that occupy widely varying thermal environments within the intertidal (Sanders *et al.*, 1991) and between species of the intertidal mussel genus *Mytilus* that have distinct biogeographical distributions (Hofmann and Somero, 1996). To evaluate the applicability of hsps as indicators for ecologically important heat stress it is necessary to understand how these interspecific and acclimation-induced variations in the heat-shock response are regulated.

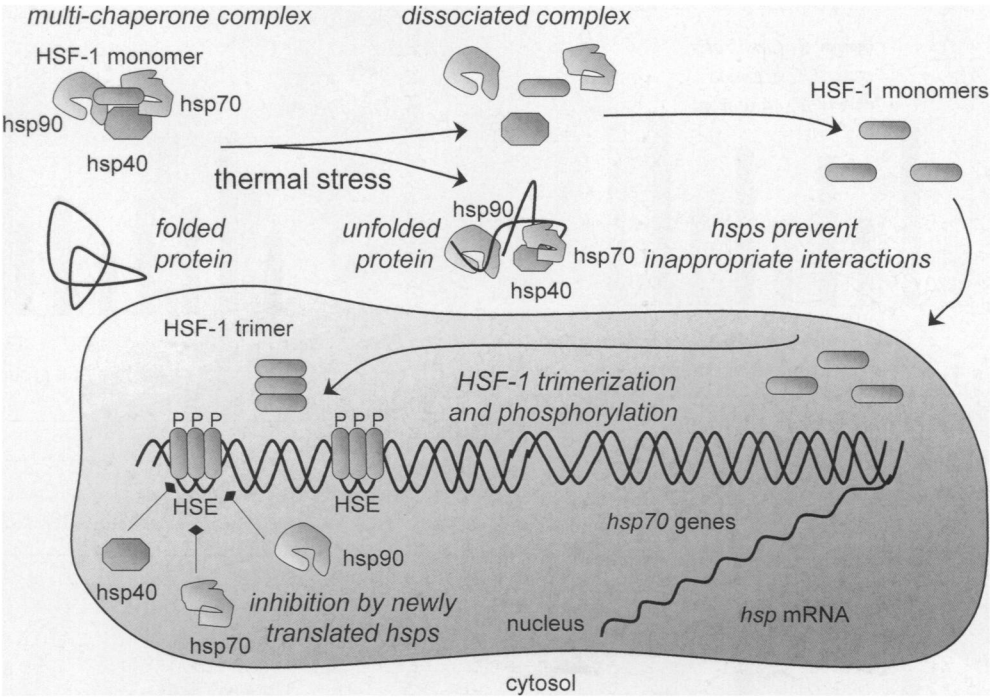

FIG. 3. The regulatory model for the transcriptional activation of the *de novo* synthesis of heat-shock proteins (hsps). Under non-stressful conditions, heat-shock factor 1 (HSF1) monomers are associated with at least hsp70, hsp40 and hsp90 (for references see Tomanek and Somero, 2002). During stressful conditions hsps prevent proteins from denaturing and the complex between HSF1 and hsps dissociates. Free HSF1 monomers can form active trimers, which bind to the heat-shock element (HSE) and are transcriptionally competent after phosphorylation. As hsp levels increase, they re-associate with HSF1 and thereby lead to the repression of *hsp* gene expression (modified after Tomanek and Somero, 2002).

REGULATION OF INTERSPECIFIC AND ACCLIMATION-INDUCED VARIATION

I hypothesized that the interspecific and acclimation-induced variation in T_{on} of hsp70 synthesis is regulated through the interaction between HSF1, a transcription factor that binds specifically to heat-shock elements (HSE), which are located in the promoter upstream of *hsp* genes, and several hsps, among them the two known putative main repressors of the response, hsp70 and hsp90. This interaction is at the core of a model of transcriptional regulation of hsp expression that is called the "cellular thermometer" (DiDomenico *et al.*, 1982; Craig and Gross, 1991; Lindquist, 1933; Morimoto, 1998). This model assumes that several hsps, *e.g.*, hsp70, hsp40 and hsp90, keep HSF1 inactive by binding to the monomer under non-stressful conditions (Fig. 3). These hsps bind to unfolding proteins during stressful conditions and therefore 'free-up' the monomer to allow formation of an active trimeric form that can bind to the HSE. Although my studies concentrated on the presumed equilibrium between a multi-chaperone complex that consists of several hsps and HSF1. There are several additional regulatory steps downstream from this interaction that are likely to contribute to interspecific differences in the heat-shock response (Morimoto, 1998; for a more detailed description see Introduction in Tomanek and Somero, 2002).

Regulation of interspecific differences

The model predicts that relatively higher levels of hsp70 and hsp90 should inhibit an early onset of hsp expression and therefore cause a higher T_{on}, assuming that levels of HSF1 stay equal. Higher levels of HSF1, however, should allow for an earlier onset of the response because free HSF1 monomers not complexed with hsps can form active trimers at lower temperatures, assuming that levels of hsps and other protein stabilizing molecules, *e.g.*, compatible osmolytes, stay equal.

When I compared endogenous levels of two hsp70 isoforms, hsp90 and HSF1 in laboratory-acclimated snails of all three temperate species I found that interspecific and acclimation-induced changes in hsp72, a low-molecular-mass hsp70 isoform, seemed to explain the variation in T_{on} (Figs. 1 and 2) in accordance with the regulatory model (Tomanek and Somero, 2002). For example, the low- to mid-intertidal zone *T. funebralis* showed higher levels of hsp72 following acclimation to 13°C and 18°C than the two lower occurring *Tegula* species (Fig. 4A). These relatively higher endogenous levels corresponded with the higher temperature at which the synthesis of hsps was activated (T_{on}) in this species at these acclimation temperatures (Figs. 1 and 2). Following acclimation to 23°C, however, all three species showed equal levels of hsp72 and induced the response at a common temperature of 27°C

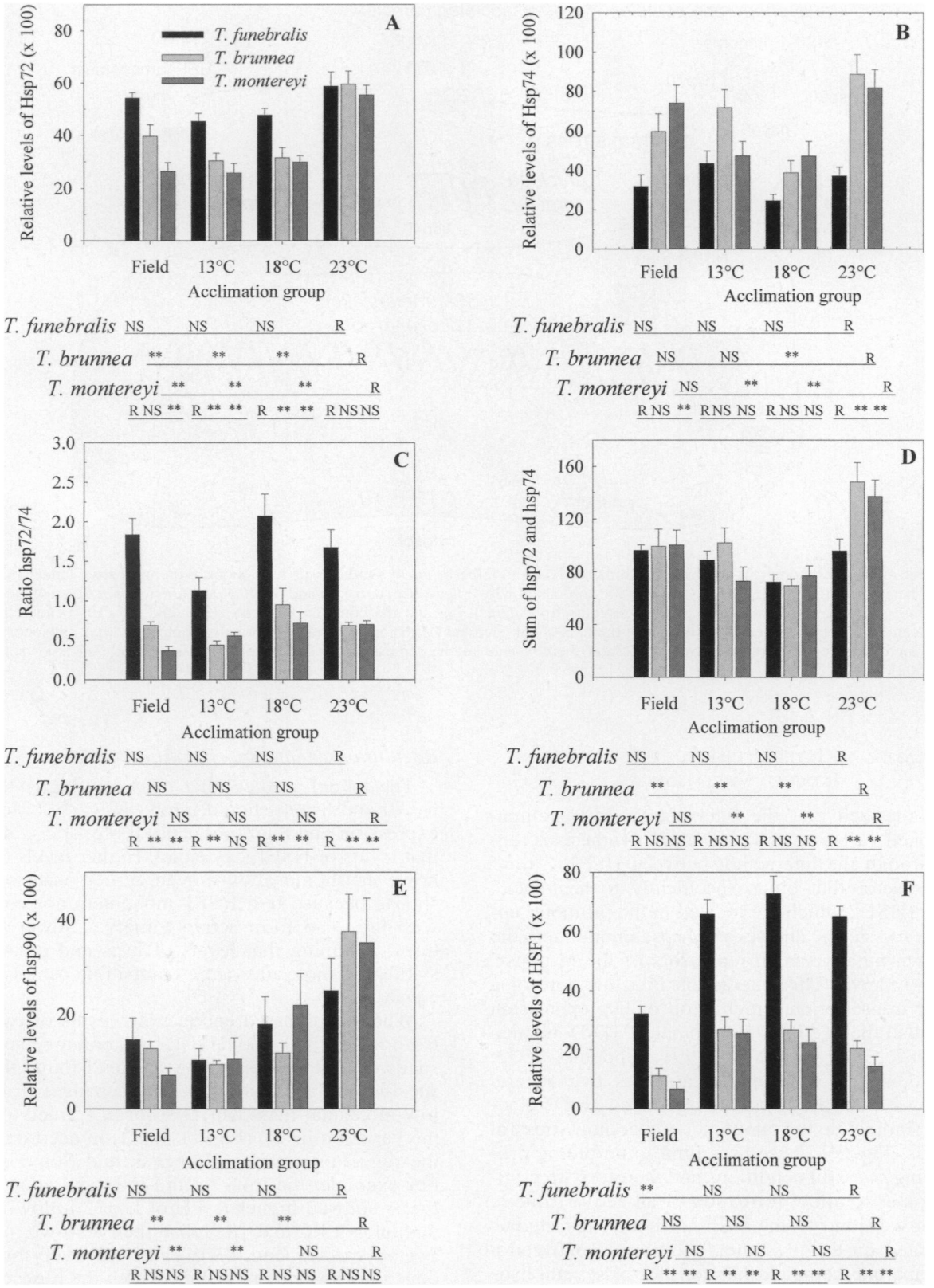

FIG. 4. Endogenous levels of hsp72 (A), hsp74 (B), the ratio of hsp72/74 (C), the sum of hsp72 and hsp74 (D), hsp90 (E) and HSF1 (F) relative to an internal control in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13°C, 18°C and 23°C for 30 to 34 d. Long lines indicate pairwise comparisons within species among treatments; short lines indicate comparisons within treatments among species. R refers to the group that all other groups along a line are compared to. P-values: **<0.05; NS = non significant. Data are means ± 1 S.E.M. (n = 5 for all data points except for *T. brunnea* at 18°C (n = 4) in case of A, C and D; and for *T. funebralis* under field conditions (n = 4) and at 13°C (n = 3) and 18°C (n = 4) and *T. montereyi* at 18°C (n = 4) and 23°C (n = 4) in case of F. For statistical analysis and further details see Tomanek and Somero, 2002).

(Figs. 2 and 4A). Hsp74, another hsp70 isoform, and hsp90 did not show such interspecific differences (Figs. 4B, E). I also considered that both hsp72 and hsp74 together may directly interact with HSF1 and, thus, that the sum of both would differ between congeners (Fig. 4D). Such differences did exist at warmer (23°C) but not at colder acclimation temperatures and therefore did not correlate with interspecific patterns in T_{on}.

Thus, so far it seemed as if the regulatory model with the interaction between HSF1 and several hsps at its core could explain interspecific variation in T_{on}, but this assumed equal levels of HSF1 between species. However, solid-phase immunoassays (western analysis) showed that HSF1 levels were more than twice as high in *T. funebralis* than in the lower occurring species, *T. brunnea* and *T. montereyi* (Fig. 4F). These unequal levels did not change with acclimation to warmer temperatures. The cellular thermometer model predicts that higher levels of HSF1, as found in *T. funebralis*, would induce the stress response at comparatively lower temperatures; instead, *T. funebralis* induced hsp synthesis at higher temperatures than the subtidal species. Even if I account for the differing levels of hsp72 by calculating the ratio of hsp72/HSF1 as a measure of the repression of stress response activation, *T. funebralis* still displays lower ratios and is therefore less repressed than the two subtidal species (data not shown). This led me to conclude that the model for the transcriptional regulation of hsp expression, with a multi-chaperone complex binding to the HSF1 at its core, cannot explain the variation in T_{on} between species.

Regulation of intraspecific differences

Within a species, shifts in T_{on} did correlate with increasing levels of hsp72, hsp74, the sum of both, and hsp90 for both subtidal *Tegula* species (Fig. 4). HSF1 levels did not change significantly during acclimation to warmer temperatures. In *T. funebralis* neither T_{on} of hsp70 synthesis nor endogenous levels of hsp72, hsp74, the sum of both, or hsp90 changed with acclimation. As studies of intertidal fish of the genus *Gillichthys* and mussels of the genus *Mytilus* (Dietz and Somero, 1992; Roberts *et al.*, 1997) indicated, intraspecific changes in T_{on} of hsp synthesis due to field-acclimatization or laboratory-acclimation are likely to be regulated by hsps, *e.g.,* hsp70 and hsp90, that have been shown to directly interact with the transcription factor HSF1 Thus, the cellular thermometer model may be applicable for explaining intraspecific acclimation-induced changes in T_{on}.

Distinguishing between functionally different hsp70 isoforms

Considering that hsps can function either as folding chaperones during normal protein translation or as stress proteins during protein denaturing conditions, I was interested in analyzing the ratio of the two hsp70 isoforms I detected. From previous work I knew that the lower molecular-mass isoform, (hsp72 in *Tegula*), was strongly heat-inducible in a variety of marine invertebrates (Hofmann and Somero, 1995; Roberts *et al.*, 1997). In contrast, the higher molecular-mass isoform, (hsp74 in the case of *Tegula*), changed little with various laboratory-acclimation or field-acclimatization conditions and seemed to be constitutively expressed without responding immediately to thermally stressful exposures. Plotting the ratio of hsp72 to hsp74, I discovered a strong contrast between species, with ratios being more than twice as high in *T. funebralis* than in *T. brunnea* and *T. montereyi* (Fig. 4C). If, as I hypothesize, hsp72 and hsp74 are indicators for levels of protein synthesis and the requirement to repair damaged proteins, respectively, these contrasting ratios certainly seem plausible in terms of the thermal stress that I suspect these species to experience in their respective environments. In addition, such differences in the allocation of hsp70 isoforms that differ in function parallel the greater levels of HSF1 in *T. funebralis*. These results indicate that *T. funebralis* has increased its "safety margins" towards ameliorating potentially higher levels of stress-induced protein damage. Clearly, the higher levels of both hsp72 and HSF1, first, protect cells from heat stress and, second, predispose cells to activate the energetically costly stress response that also, because of the preferential synthesis of hsps, disrupts protein homeostasis (Tomanek and Somero, 1999). What may be the ecological consequences of upregulating the safety margins? A comparison of estimated growth rates for *T. funebralis* and *T. brunnea* as well as *T. montereyi* showed that the subtidal species grow about two to three times as fast as the intertidal *T. funebralis* (Frank, 1965; Paine, 1969; Watanabe, 1982). This suggests that a greater tolerance to heat stress and the increased requirement to repair damaged proteins that are associated with a stressful environment may be costly.

Ecologically Relevant Variation in Hsp70 Expression Under Natural Conditions

I am ultimately interested in the role of the observed variation in hsp expression in setting limits to the vertical distribution ranges of *Tegula* congeners. Several of the results from my laboratory-acclimation experiments led me to hypothesize (i) that field-acclimatized mid-intertidal *T. funebralis* should respond to stressful low tide periods by increasing endogenous levels of the heat-inducible hsp70 isoform (hsp72), (ii) that the subtidal to low-intertidal *T. brunnea* could be close to its thermal limits if transplanted into the mid-intertidal zone and (iii) that the latter species would also synthesize increasing (and higher than in *T. funebralis*) amounts of hsp70 under these conditions. I tested these predictions by transplanting *T. brunnea* from the low- into the mid-intertidal zone, using cages that were either directly exposed to the sun (except for a minor shading effect due to the metal mesh) or protected from the sun and from which I collected specimens every three to four days for a month long time period

(L. Tomanek and E. Sanford, unpublished data). To better compare species, I included a control of caged *T. funebralis* (sun-exposed) and collected specimens from their natural field sites (from low-intertidal sites in case of *T. brunnea* and mid-intertidal sites in case of *T. funebralis*). To evaluate the effect of thermal stress I measured body temperatures of snails from the field (for method see Tomanek and Somero, 1999).

Several comparisons (Fig. 5) highlight the effect transplanting had on the expression of hsp72 (one of the two hsp70 isoforms) in the various experimental groups. First, expression of hsp72 (and hsp74, data similar to Fig. 5) differed distinctly between sun-exposed *T. brunnea* and *T. funebralis* during the month long sampling period. On several days *T. brunnea* had 3–6 times the amount of hsp72 (and hsp74) in comparison to *T. funebralis*. Two of these collections were made following a period of several low-tide exposures (4/03/2000 and 4/13/2000). The third remarkable increase (4/21/2000) of hsp72 (and hsp74) in *T. brunnea* was not preceded by extreme low-tides but instead by a great increase in the daily temperature range. Following a correlation analysis I found that levels of hsp72 and changes in daily temperature range were significantly correlated with a lag of four days in the transplanted and sun-exposed *T. brunnea*, indicating that a change in endogenous levels of hsp72 follows a change in daily temperature range (see hsp72 levels following an increase in range by 7°C, Fig. 5B, C) after at least four days. Within our sites I observed heterogeneous survival rates for *T. brunnea*, depending on the particular microhabitat the cages were located at, but no mortality for *T. funebralis*, suggesting that all sun-exposed *T. brunnea* experienced thermal stress close or beyond their thermal limits (data not shown).

Second, sun-exposed *T. brunnea* responded with a greater increase in levels of hsp72 (and hsp74) than transplanted but sun-protected and field-collected specimens (Fig. 5D). Sun-protected treatments in turn showed slightly greater levels of hsp72 (but not of hsp74) only during the first ten days and were subsequently similar to field-collected specimens.

Third, field-collected specimens of both species did change little and differed only slightly in levels of hsp72, with *T. funebralis* expressing higher amounts during the first eight days and *T. brunnea* expressing higher levels during the last fourth of the month.

From these data we conclude (i) that the thermal stress that *T. funebralis* experienced in the field and in a sun-exposed caged control group during this period, which included three midday low-tide periods, was not severe enough to increase levels of hsp72 and hsp74 (data not shown but similar to Fig. 5), at least not enough to maintain a signal over the three to four day sampling interval. However, on several days the collection of samples was preceded the day before by a midday low-tide that raised the temperature of snails (likely in both sun-exposed and field-collected specimens) to up to 30°C and higher (04/03/2000 and 04/13/2000). If hsps were synthesized in response to such thermal exposures we should have detected increasing levels within the next 24 hr. (ii) Mortality at least in some of the sun-exposed cages suggests that *T. brunnea* being transplanted into the mid-intertidal zone experienced thermal stress close to its thermal limits and, (iii), it responded to this stress by synthesizing 3 to 6 times higher levels of hsp72 and hsp74 than caged and sun-exposed *T. funebralis*. It seems unlikely that a reduction in immersion (and feeding) time could have caused this response, mainly because the sun-protected group, which experienced temperatures about 4°C lower than the sun-exposed animals at the same height in the intertidal zone (data not shown), did only differ from the field-collected specimens inconsistently (Fig. 5D).

Is Hsp70 an Indicator for Ecologically Important Heat Stress?

The results of the studies discussed above complement each other and provide a broad, yet still incomplete, picture of the importance of hsps as indicators for ecologically critical sublethal heat stress. The differences between species in the *de novo* synthesis of hsps, *e.g.,* onset (T_{on}), peak (T_{peak}) and upper (T_{off}) temperature as well as the time course of hsp synthesis, in combination with the differing expression of endogenous levels of hsp72 and hsp74 in transplanted animals, suggest that the interspecific variation in the stress response prevents the subtidal to low-intertidal *T. brunnea* and *T. montereyi* from inhabiting the thermal environment of the mid-intertidal *T. funebralis*. In turn, the wider thermal range of protein synthesis in the mid-intertidal *T. funebralis* (and of the subtropical *T. rugosa*) appears to be an adaptation for coping with a thermally more variable environment.

Within a species it appears that only heat stress that is very close to the upper thermal limits of an organism triggers the increase of endogenous levels of hsp70. This was unexpected in light of our metabolic labeling studies that suggested that *T. funebralis* activates the response frequently under the thermal conditions it experiences in the mid-intertidal zone. Furthermore, on a seasonal basis, it was shown that intertidal goby fish of the genus *Gillichthys* and intertidal mussels of the genus *Mytilus* acclimatized to changing ambient temperatures by shifting their induction of the response (T_{on}) as well as by changing endogenous levels of hsps (Dietz and Somero, 1992; Roberts, *et al.*, 1997). It is surprising that low-tide periods that certainly impose greater thermal fluctuations to intertidal animals than seasonal changes, although only for short time periods, do not trigger the same changes (at least not in regard to endogenous levels of hsp70). These discrepancies may be resolved in the future by distinguishing between constitutively expressed hsps that function during protein synthesis under non-stressful conditions and hsps, which are heat-inducible and constitutively expressed, that are preventing proteins from further denaturing under stressful conditions. My re-

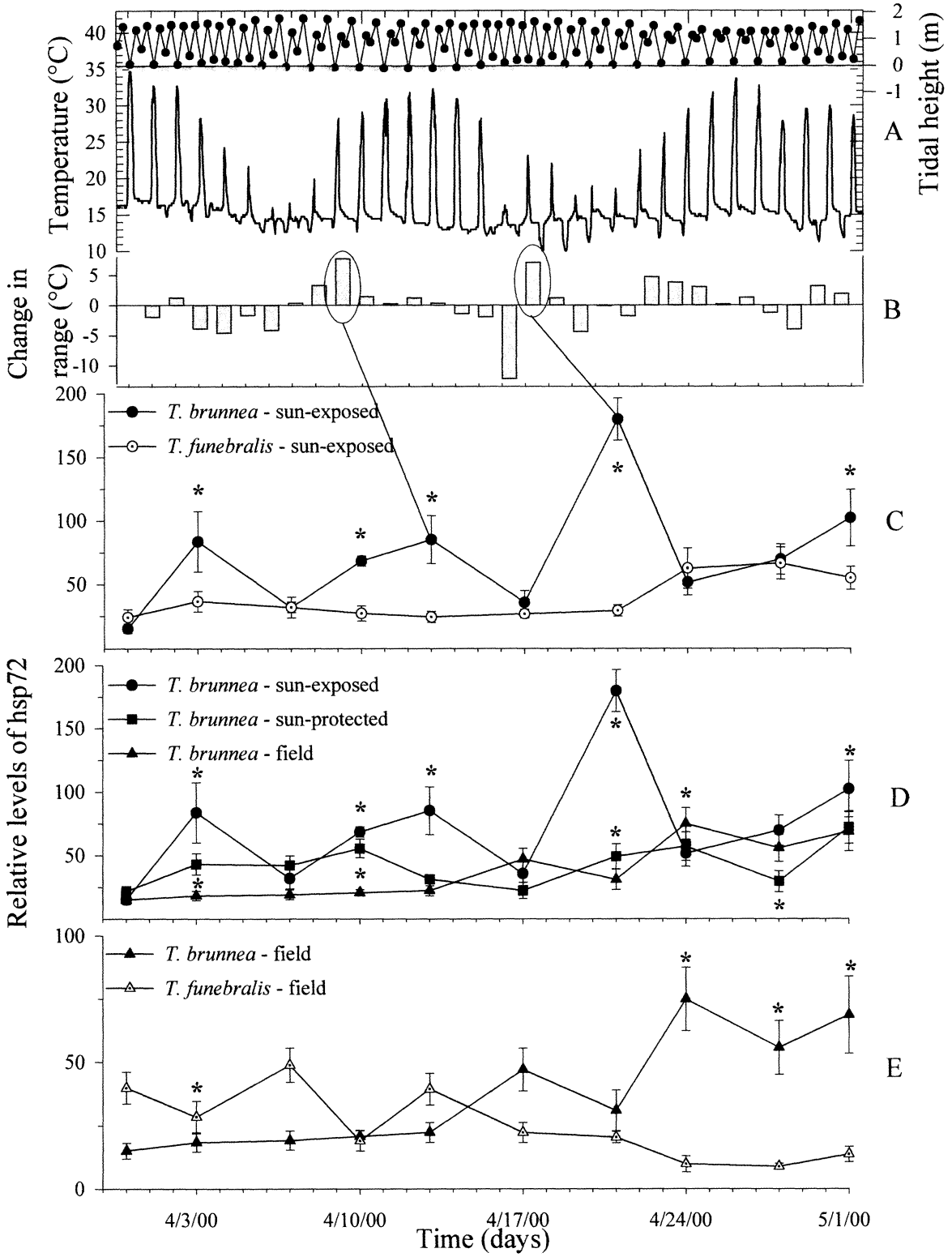

FIG. 5. Time course of endogenous levels of hsp72 in (C) caged and sun-exposed *T. funebralis* (low- to mid-intertidal zone) and *T. brunnea* (subtidal to low-intertidal zone) that were transplanted into the mid-intertidal zone (from 31/03/2000 to 05/01/2000), in (D) field-acclimatized, caged sun-exposed and sun-protected *T. brunnea* (with field specimens collected from the low-intertidal zone), and in (E) field-acclimatized *T. funebralis* (from the mid-intertidal zone) and *T. brunnea* (from the low-intertidal zone). Levels are expressed relative to an internal control. Temperatures are from a gelatin-filled snail shell that was placed in the mid-intertidal zone (A; see Tomanek and Somero, 1999). Caged sun-exposed and sun-protected snails experienced slightly lower temperatures (about 1°C and 4°C, respectively). Shown is also the change in daily temperature range relative to the previous day (B) and two occasions that exemplify how such changes preceded increases in endogenous levels of hsp72 in sun-exposed *T. brunnea* (B and C). Tidal heights were obtained for Monterey Bay. P-values indicate if means on this date were different: *≤0.05. Values are mean ± 1 S.E.M., n = 5–7 for almost all data points (L. Tomanek and E. Sanford, unpublished data).

sults indicate that the ratio of the two (hsp70) isoforms may be regulated differently in these congeners. A mechanistic explanation for such differences in regulation is certainly going to involve interspecific changes at several levels of the stress response activation cascade (Fig. 3). However, these results remind us that the value of an indicator of physiological state for ecological problems greatly depends on a mechanistic understanding of the physiological process in question.

In summary, we may have to be more careful in interpreting intraspecific changes in endogenous levels of hsp70 and other hsps, especially if we do not separate isoforms by function, as direct indications for heat stress. However, it seems apparent that interspecific variation in the heat-shock response contributes to limit the upper vertical distribution range of rocky intertidal *Tegula* congeners. Finally, the studies presented here promise that the *Tegula* genus could provide an ideal phylogenetic group to study other biochemical and molecular adaptations to the rocky intertidal environment (Hochachka and Somero, 2002).

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